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Effects of dietary supplementation of a commercial prebiotic Previda® on survival, growth, immune responses and gut microbiota of Pacific white shrimp, *Litopenaeus vannamei*

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Abstract

A 35-day feeding trial was conducted to evaluate growth, bacterial populations of the gastrointestinal tract (GIT) and immune responses of *Litopenaeus vannamei* fed diets containing the commercial prebiotic Previda®. Diets were formulated to contain Previda® at 0, 0.2, 0.5, 1.0 or 1.6 g kg^{-1} by weight. At the end of the study, differences in weight gain and survival among treatments were not significant ($P > 0.05$), but denaturing gradient gel electrophoresis analysis revealed that the microbial communities in the GIT changed significantly with the inclusion of dietary Previda® at different levels. Previda® was therefore able to selectively modify the microbial communities in the shrimp's GIT. Although individual bacterial species were not identified, enteric populations in shrimp fed the prebiotic at similar levels of inclusion were genetically similar. In addition, shrimp fed Previda® at 1.6 g kg^{-1} responded significantly ($P < 0.05$) better immunologically with respect to hemocyte phagocytic capacity, haemolymph protein, hyaline cell counts and haemolymph glucose compared with shrimp fed the basal diet. Although shrimp were not exposed to virulent pathogens in this study, the observed upregulation of some of immune responses upon prebiotic supplementation indicates that an improved outcome of such challenges may be anticipated in Previda®-fed shrimp under commercial conditions.

KEY WORDS: gut microbiota, innate immunity, prebiotics, shrimp

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Introduction

Shrimp farming is one of the most profitable aquaculture sectors in the world, but the industry has faced several setbacks due to ravaging bacterial and viral diseases (Smith *et al.* 2003). It is in recognition of the severity of epizootics events that the World Bank recommended investing $275 million in targeted research for the period between 1996 and 2010 with the hope of offering innovative solutions, as the previous methods of disease control have proven inadequate (Smith *et al.* 2003). The use of antibiotics in production has been criticized due to the potential development of antibiotic-resistant bacteria, the presence of antibiotic residues in seafood, destruction of the microbial population in the aquatic environment and suppression of the animal's immune system (Ng *et al.* 2009). As an alternative, it has been suggested that targeted modifications to bacterial populations in the gastrointestinal tract (GIT) of shrimp and other aquatic organisms may hold the key to optimizing weight gains (WG) and improving health status of farmed animals. Such

modifications would involve favouring bacterial populations known to be symbionts or commensals in the shrimp's GIT, while inhibiting those that are potentially pathogenic (Lara-Flores *et al*. 2003). It is now well established that the management of the enteric microbiota to obtain these results can be achieved through the use of diet additives such probiotics, prebiotics, organic acids and their salts (Mahious *et al*. 2006; Li *et al*. 2007; Zhou *et al*. 2007; Lückstädt 2008; Wang *et al*. 2008; Ringø *et al*. 2010; Anuta *et al*. 2011).

The usefulness of prebiotics in aquaculture is well documented (Li *et al*. 2004a,b, 2007). Prebiotics also have been used to enhance growth through improved gut health in fish (Kihara *et al*. 1995; Refstie *et al*. 2006; Ringø *et al*. 2006; Torrecillas *et al*. 2007). One potential mechanism for this improvement, common to terrestrial and aquatic organisms, is the colonization of the enteric milieu by beneficial bacteria (e.g. *Lactobacillus* sp.), thus competitively excluding potentially pathogenic microorganisms (Bailey *et al*. 1991; Probert *et al*. 2004; Buentello *et al*. 2010).

In fish, various reports have documented improvements following dietary supplementation with commercially available prebiotics in feed efficiency (FE), enhancement of non-specific immune responses for species such as hybrid striped bass (*Morone chrysops* x *M. saxatilis;* Li & Gatlin 2004), rainbow trout (*Oncorhynchus mykiss;* Staykov *et al*. 2007) and Atlantic salmon (*Salmo salar;* Grisdale-Helland *et al*. 2008).

Shrimp culture also has benefited from prebiotic applications. Zhou *et al*. (2007) evaluated the effects of dietary short-chain fructooligosaccharides (FOS) in *L. vannamei*, observing WG and feed conversion ratio (FCR) improvements as well as displacement of potentially pathogenic bacteria in the GIT by mostly *Lactobacillus* sp. known to be beneficial to the health of shrimp. Genc *et al*. (2007) reported enhanced WG and FCR when the green tiger prawn, *Penaeus semisulcatus,* was fed diets containing mannanoligosaccharides (MOS). However, a more thorough examination of data published to date on the use of prebiotics in shrimp feeds points to lack of effects (Li *et al*. 2009), inappropriate inclusion levels (Smith *et al*. 2003), growth suppression (Sajeevan *et al*. 2006) and overall, a general inconsistency in the results obtained. These erratic results from dietary prebiotic interventions in shrimp call for specific studies to help clarify the issue. Therefore, the present experiment was conducted to determine the optimal inclusion rate of the commercially available prebiotic Previda® and to evaluate its effects on the survival, growth, innate immune response and gut microbiota of juvenile Pacific white shrimp, *Litopenaeus vannamei*.

Materials and methods

Experimental diets

The composition of the experimental diets is fully described in Table 1. The basal diet included cholesterol, vitamins and mineral premixes to meet the known nutritional requirements of penaeid shrimp (D'Abramo *et al*. 1997). The vitamin and mineral premixes identified as #1 and #2 are proprietary products of Ziegler Bros, Gardner, PA, USA. A commercial prebiotic Previda®, manufactured by Temple Inland Inc. (Diboll, TX, USA) and now marketed

Table 1 Composition of experimental diets (g kg^{-1})

Ingredient	Basal	0.2	0.5	1.0	1.6
Menhaden Fishmeal[1]	8.0	7.98	7.96	7.92	7.87
Squid Muscle[1]	30.0	29.9	29.9	29.7	29.5
Soybean meal, 48% CP, S. ext.[1]- 90%	5.70	5.7	5.7	5.6	5.6
Phospholipid[2]- 97%	4.0	3.99	3.98	3.96	3.94
Wheat starch[3]	29.5	29.4	29.4	29.2	29.0
Mineral/vit premix[4]#1	0.25	0.25	0.25	0.25	0.25
Mineral/vit premix[4] #2	0.21	0.21	0.21	0.21	0.21
Vitamin C[4]	0.04	0.04	0.04	0.04	0.04
Diatomaceous earth[5]	2.80	2.79	2.79	2.77	2.76
Alginate[6]	2.0	2.0	2.0	2.0	2.0
Sodium chloride	0.70	0.70	0.70	0.70	0.70
Potassium chloride	1.90	1.90	1.89	1.88	1.87
Magnesium oxide	1.60	1.60	1.59	1.58	1.57
Calcium carbonate	2.50	2.50	2.49	2.48	2.46
Dicalcium phosphate	4.20	4.19	4.18	4.16	4.13
Previda[7]	0.0	0.20	0.50	1.0	1.60
Sodium hexametaPO$_4$	1.0	1.0	1.0	0.99	0.98
Cholesterol	0.20	0.20	0.20	0.20	0.20
Cellulose[5]	3.20	3.19	3.18	3.17	3.16
Oil, menhaden[8]	0.60	0.60	0.60	0.60	0.60
Oil, soybean[9]	0.60	0.60	0.60	0.60	0.60
Chromic oxide	1.00	1.00	1.00	1.00	1.00
Analysed proximate composition of two experimental diets (g kg^{-1} as fed)					
Moisture		9.93			
Ash		17.2			16.9
Crude protein		36.4			37.0
Crude fat		6.6			6.6
Crude fibre		1.3			1.4
Mineral analysis					
Phosphorus		1.78			1.74
Calcium		2.49			2.48

[1] Oceanic Institute, Waimanalo, HI, USA.
[2] Cargill.
[3] MP Biomedicals, Solon, OH, USA.
[4] Zeigler Bros., Gardners, PA, USA.
[5] Sigma- Aldrich, St Louis, MO, USA.
[6] ChemPoint, Bellevue, WA, USA.
[7] Temple Inland, Diboll, TX, USA.
[8] Omega Protein, Houston, TX, USA.
[9] IGA Family Center, Port Aransas, TX, USA.

by Novus International (Saint Charles, MO, USA), was incrementally added to the basal diet to obtain concentrations of 0, 0.2, 0.5, 1.0 and 1.6 g kg^{-1} by dry weight. Previda® is 100% hemicellulose comprised of 83% sugars out of which 71% are in an oligomeric form. There is 5% ash in the product, while other materials naturally present in the hemicellulose extract make up 12%. Diets were prepared by the method of Gong *et al.* (2000). Briefly, dry ingredients (excluding alginate and sodium hexametaphosphate) were added to a Hobart L-800 mixer (Hobart Corporation, Troy, OH, USA) and thoroughly blended. A portion of this mix was then combined with the remaining dry ingredients in a V-mixer (3 L; Patterson Kelley, PA, USA) and blended again. Menhaden fish oil and soybean oil (Virginia Prime; Omega Protein, Houston, TX, USA and Crisco pure soy oil, respectively) were added, and the resulting mixture was thoroughly blended. This base mix and Previda® were subsequently combined in the V-mixer in different proportions to attain the desired level in each experimental diet. Hexametaphosphate and alginate were added to obtain uniform dough, which was pelletized through a 2-mm die (Hobart A- 200 meat grinder). Strands of diets were crumbled and allowed to dry (with air circulation) at 30 °C overnight. Diets were subsequently ground to the appropriate size, packed in sealed plastic bags and stored in the freezer at −20 °C until fed.

Experimental shrimp and feeding

Postlarval *L. vannamei,* obtained from Harlingen shrimp farms, Harlingen, TX, were reared at the Texas AgriLife Research Mariculture Laboratory at Port Aransas, TX. Rearing conditions were maintained at 30.3 ± 0.38 °C water temperature, 28.6–35.9 ppt salinity and 5.6 ± 1.1 mg L^{-1} dissolved oxygen. Shrimp were fed a commercial postlarval diet (Rangen Inc., Angleton, TX, USA). Within the rearing receptacles, the commercial diet was supplemented with live *Artemia* nauplii twice daily until the feeding trials were initiated (2 weeks). This study was conducted in an indoor, temperature-controlled, recirculating aquaculture system with minimal natural productivity. Sea water was pumped through a combination sand, biological and 50-μm cartridge filters and UV disinfection units set up to achieve a recirculation rate of 40 complete water exchanges per tank per day. Forty rectangular tanks, each approximately 30 L (bottom area 0.1 m^2), were used for the study. Groups of six shrimp of similar size (~0.2 g initial weight) were blotted dry and weighed before being stocked into each tank. Mean initial weight of shrimp did not differ more than 3% among tanks. There were eight replicate groups of shrimp for each dietary treatment. At the end of the study, surviving shrimp from each tank were dried in the same manner and group-weighed. Automatic feeders were used in the study and ensured that shrimp were fed 15 times per day. Feeding rate was maintained so that the juvenile shrimp were fed to slightly exceed satiation. Shrimp were monitored daily for moulting activity and survival. Uneaten diets and exuviae were syphoned out daily prior to the first morning feeding. Water quality variables such as temperature, salinity and dissolved oxygen were monitored daily. Total ammonia nitrogen (N), nitrate-N and nitrite-N were monitored weekly using methods described by Spotte (1979). The experiment was terminated after a 35-day feeding period.

Intestinal tract samples, DNA isolation and PCR

At trial termination, the intestinal tract contents (sampled shrimp) immediately posterior to the stomach from three animals per tank were aseptically removed and pooled per tank, flash-frozen in liquid N and stored at −80 °C until characterization of autochthonous microbial populations. Genomic DNA was isolated from 0.2 g of freeze-dried digesta with the QIAamp DNA Mini Kit (Cat# 51304; Qiagen, Valencia, CA, USA) using the manufacturer's protocol with the following modifications: the pellets were suspended in 300 μL of the genomic lysis solution containing lysozyme (20 mg mL^{-1}, L- 687 Sigma Chemical Co., St. Louis, MO, USA) and mixed with sterile pestles. The solution was incubated at 37 °C for 2 h after which it was centrifuged at 20 800 ***g*** for 3 min and the supernatant was removed and placed in a sterile 1.5-mL microcentrifuge tube. To this solution was added 1.5 μL of RNAse A (4 mg mL^{-1}) and the mixture was incubated at 37 °C for 45 min. Twenty microlitres of proteinase solution, supplied by the manufacturer and according to instructions, was added, followed by vortexing and incubation at 56 °C for 30 min. The solution was incubated at 95 °C for 15 min. After DNA precipitation, hydration and quantification, polymerase chain reaction (PCR) was subsequently conducted using the method of Hume *et al.* (2003). Briefly, bacteria-specific PCR primers (forward and reverse) were used to target conserved regions flanking the variable V3 region of 16S rDNA.

Denaturing gradient gel electrophoresis and dendrogram analysis

Denaturing gradient gel electrophoresis (DGGE) was conducted following the method of Hume *et al.* (2003) as

modified by Li *et al.* (2007). The fragment analysis of pattern relatedness was determined with Molecular Analysis fingerprinting Software (v1.6; Bio-Rad, Richmond, CA, USA). The unweighted pair group method with arithmetic mean algorithm was used to construct a dendogram of DGGE band profiles. The dendogram analysis compares DGGE profiles based on numbers, positions and intensities of bands. The dice percentage similarity coefficient (SC) was used for computing sample similarity based on band position and intensity. Comparisons between sample band patterns are expressed as a percentage SC (Fig. 1).

Immunity and physiological assays

At the termination of the feeding trial (week 5), eight shrimp (1 per replicate tank) were sampled only from the groups fed the lowest, medium and highest levels of Previda® (0, 0.5, and 1.6 g kg^{-1}, respectively) for stress and immunity evaluations. We chose to conduct immunity assays only on shrimp fed the lowest, the highest and medium levels of Previda®. This strategy maximizes our power of interpretation on the effects of Previda® on shrimp's immunity while minimizing expenditure. Sampling at both ends of the spectrum and including a medium data point is also appropriate for regression fitting. About 100 μL of haemolymph was withdrawn from the ventral sinus of each shrimp into a 1-mL sterile syringe using a 25-gauge needle containing 0.5 mL of anticoagulant (30 mM trisodium citrate, 0.34 M sodium chloride, 10 mM EDTA, pH 7.55, osmolarity 780 mOsm kg^{-1}). For physiological parameters, a second batch of haemolymph (about 100 μL) was obtained using the same procedure, but no anticoagulant was added. Immediately after obtaining the EDTA-treated haemolymph, 25 μL was removed and mixed with 25 μL of trypan blue in microcentrifuge tubes, mixed thoroughly and then placed in a hemocytometer for cell counting. Hemocytes were identified following the guidelines of Braak (2002). Both total (THC) and differential (DHC) hemocyte counts were obtained. To determine the phagocytic activity of macrophages (MØ), a microscopic counting technique as described by Brown *et al.* (1996) and modified by Mustafa *et al.* (2000) was followed. This assay determined the proportion of hemocytes that were able to engulf formalin-killed bacteria, *Bacillus megaterium*. A culture of *B. megaterium* was grown in the laboratory in a 5-mL tube of tryptic soy broth at 37 °C for 18–24 h. After incubation, 5 mL of formaldehyde was added to the solution and mixed. One hundred microlitres of this killed culture was added to double-etched microscope slides (Eric Scientific, Portsmouth, NH, USA) containing attached hemocytes that had been isolated from the sampled shrimp and incubated for 90 min at room temperature. Upon the addition of these bacteria, slides were incubated for another 60 min.

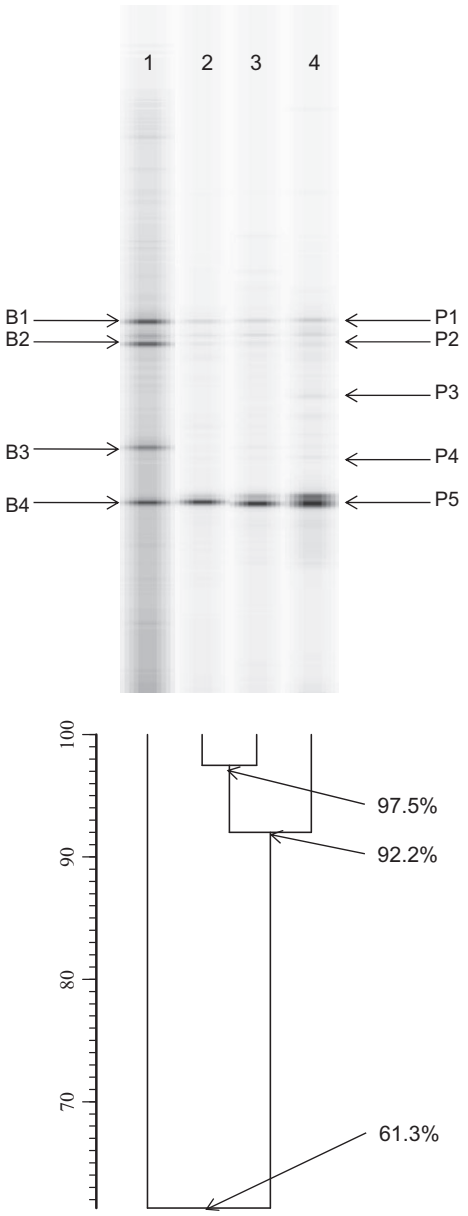

Figure 1 Dendrogram of the gastrointestinal tract (GIT) microbial communities of shrimp, *Litopenaeus vannamei*, fed the basal diet (lane 1), and diets containing Previda® at 0.2, 0.5, 1.0 and 1.6 g kg^{-1}, lanes 1, 2, 3 and 4, respectively. The community from shrimp fed the basal diet (B1-4) was different from that of shrimp fed the Previda® diets [similarity coefficient (SC) = 61.3%]. The GIT tract microbial community from shrimp fed the Previda® diets was very similar (SC = 92.2%), but only the communities of shrimp fed 0.2 and 0.5 g kg^{-1} Previda diets were identical (lanes 2 and 3, SC > 95%). Arrows point to important banding patterns.

Following incubation, the slides were washed with phosphate-buffered saline (PBS), air-dried, fixed in 100% methanol and stained with Wright-Giemsa stain (Sure-stain, Fisher Diagnostics, Middletown, VA, USA) for microscopic examination at 100×. At least 100 cells were examined to determine the phagocytic capacity of shrimp hemocytes (the per cent of hemocytes containing five or more bacteria; Enane *et al*. 1993). Due to the volume of samples, we counted and analysed live cells for shrimp obtained from three data points – basal diet, 0.5 and 1.6 g kg^{-1} levels of Previda®.

Haemolymph glucose was measured following protocols previously described by Schreck & Moyle (1990) and Gensic *et al*. (2004) and validated for the use in fish by Wedemeyer *et al*. (1990). Briefly, a drop of EDTA-free haemolymph was placed on a glucose strip and inserted into a standard glucometer (Precision Xtra; Abbot Laboratories, Abbot Park, IL, USA). For packed hemocyte volume, EDTA-free haemolymph was collected in capillary tubes, centrifuged with Critspin Micro-Hematocrit Centrifuge (StatSpin Inc., Westwood, MA, USA) and read using a Micro-Hematocrit Capillary Tube reader (Monojet Scientific, St Louis, MO, USA). Haemolymph protein was estimated using a hand-held protein refractometer (VEEGEE; Lab safety Supply Inc. Janesville, WI, USA).

Statistical analysis

Data obtained for immune studies were statistically analysed using Minitab 15.1.0 (2007). Means and standard errors of means were calculated for each assay by one-way analysis of variance (ANOVA), and differences were considered significant when $P < 0.05$. A post-ANOVA comparison of multiple means test (Tukey's) was performed for mean separation among treatments. Growth and performance data were analysed using one-way ANOVA followed by Student–Newman–Keuls test. Finally, immune responses of *L. vannamei* were compared via regression analysis to determine differences in response to varying levels of the prebiotic in the diet.

Results

Water quality parameters

For the duration of study, total ammonia N fluctuated between 0.09 and 0.16 mg $L^{-1} \pm 0.06$ mg L^{-1}; nitrate-N, 0.29 ± 0.27 mg L^{-1}, nitrite-N, 0.81 ± 0.69 mg L^{-1}, pH 7.99 ± 0.27; water temperature, 30.31 ± 0.38 ° C; dissolved oxygen, 4.5 and 6.6 mg L^{-1}, while salinity fluctuated between 28.6 and 35.9 ppt in all the experimental units.

Growth and survival

Shrimp exhibited growth rates between 1.5 to 1.6 g $week^{-1}$ in all treatments (Table 2). Survival of shrimp in all treatments including the control was also high and ranged between 92% and 98% for shrimp fed experimental diets with or without the prebiotic, Previda.® No significant differences ($P > 0.05$) were detected for WG, FCR or survival among all dietary treatments.

Immune responses

After 35 days of culture, hemocyte packed cell volume, total hemocyte count and granular cells in shrimp did not exhibit significant differences among dietary treatments (Table 3). Semigranular cells showed significant differences between basal diet and Previda® at 0.05% (Table 3). In contrast, hemocyte phagocytic capacity, haemolymph glucose, haemolymph protein and hyaline cell counts measured for Previda®-fed shrimp were significantly different ($P < 0.05$) from shrimp fed the basal diet (Table 4). Interestingly, haemolymph protein appeared to decrease as the amount of Previda® was increased in the diet. Consequently, Previda®-fed shrimp at 1.6 g kg^{-1} of diet had significantly less haemolymph protein compared with shrimp receiving lesser prebiotic concentrations.

Intestinal microbiota

Amplicon profiles for enteric bacteria from *L. vannamei* revealed a developmental progression consisting of two

Table 2 Growth and other production data of shrimp, *Litopenaeus vannamei*, fed Previda®

Diet Previda g kg^{-1}	Weight Gain g	Growth g $week^{-1}$	Survival %	FCR	Biomass g m^{-2}
Basal	7.7	1.5	95.4	1.6	456.2
0.2	7.4	1.5	93.8	1.7	427.9
0.5	7.8	1.6	91.7	1.7	444.4
1.0	7.9	1.6	97.9	1.5	479.0
1.6	7.6	1.5	95.8	1.6	448.9
ANOVA					
Pr > *F*	0.8278	0.8647	0.5434	0.8485	0.8894
Pooled S.E.	0.309	0.059	3.697	0.109	24.807

Values are means of eight replicate tanks with six shrimp per tank. Mean responses of shrimp fed diets at different levels of Previda® supplementation were not significantly different ($P > 0.05$).

Table 3 Stress and immune response[1] characteristics of shrimp, *Litopenaeus vannamei*, fed three levels of Previda®

Diet (g kg^{-1})	Total hemocyte count ($\times 10^6$ mL^{-1})	Granular cells ($\times 10^6$ mL^{-1})	Semi- granular cells ($\times 10^6$ mL^{-1})	Hemocyte packed cell volume (%)
Basal	7.74 ± 0.64	0.79 ± 0.25	1.59 ± 0.19	36.5 ± 1.20
0.5	8.56 ± 0.62	0.33 ± 0.88	0.93 ± 0.20*	36.0 ± 1.05
1.6	8.76 ± 0.88	0.47 ± 0.10	1.56 ± 0.09	36.0 ± 1.05
Analysis of variance (*P* value)	0.401	0.17	0.021	0.931
Regression coefficient (r^2)	0.774	0.204	0.041	0.010

[1] Means ± standard error (n = 8).
Means with an * are significantly different from the control ($P < 0.05$).

Table 4 Hyaline cells and others stress and immune response indicators of shrimp, *Litopenaeus vannamei*, fed diets at three levels of Previda®

Diet (g kg^{-1})	Hyaline cells ($\times 10^6$ mL^{-1})	Haemolymph glucose (mg dL^{-1})	Haemolymph protein (mg mL^{-1})	Hemocyte phagocytic capacity (%)
Basal	5.43 ± 0.49[1]	76.0 ± 4.38	157.1 ± 6.04	23.7 ± 0.72
0.5	7.14 ± 0.54*	67.0 ± 6.89	140.0 ± 8.22	27.2 ± 1.19*
1.6	6.73 ± 0.32*	51.0 ± 4.01*	130.7 ± 7.23	29.4 ± 1.35*
Analysis of variance (*P* value)	0.031	0.009	0.051	0.008
Regression coefficient (R^2)	0.296	0.091	0.067	0.051

[1] Means ± standard error (n = 8).
Means with an * are significantly different from the control ($P < 0.05$).

main groups (Fig. 1). The intestinal tract microflora DGGE band pattern of shrimp fed the diet with no Previda® (basal diet) differed markedly from that of shrimp fed the commercial prebiotic on the basis of a SC of 61.3%. The microbial communities in the gut of shrimp fed all Previda® diets were very similar (SC = 92.2%), but only those communities from shrimp fed Previda® at 0.2 and 0.5 g kg^{-1} of diet were likely identical (SC > 95%).

Discussion

Although some commercial prebiotics have been used successfully to promote growth in fish and other aquatic animals including shrimp (Li & Gatlin 2004; Torrecillas *et al.* 2007; Zhou *et al.* 2007; Gultepe *et al.* 2012), results of the present study failed to support this observation as no significant difference was established for shrimp among all dietary treatments. Li & Gatlin (2004) reported enhanced WG of hybrid striped bass upon Grobiotic®-A supplementation, while Zhou *et al.* (2007) documented that incremental levels of short-chain FOS resulted in increased specific growth rate and WG of *L. vannamei*, although survival was low. Improved fish health has not always accompanied improvements in growth or FCR following application of prebiotics. For example, Gultepe *et al.* (2012) obtained significant improvements in growth and FCR in fish supplemented with MOS, but this prebiotic had no significant effects on general fish health. Earlier, Rodrigues-Estrada *et al.* (2008) observed that MOS at 4 g kg^{-1} not only stimulated growth, haemolytic and phagocytic activity, but improved survival when fish were challenged with *Vibrio anguillarum*. In contrast, application of MOS or FOS in the diet of Atlantic salmon did not produce any significant effects on WG or FE (Grisdale-Helland *et al.* 2008). This result reflects in part different functional properties of the various prebiotic compounds available in the market. Although the composition of most prebiotics includes oligosaccharide residues, most are derived as by-products of industrial processes and thus have varied chemical compositions. In addition, inconsistencies in prebiotic research on fish and shrimp may also result from the fact that prebiotic actions are significantly influenced by the composition and

quality of the experimental diet. For instance, studies with red drum, *Sciaenops ocellatus,* demonstrate that only fish fed diets in which 50% of the dietary protein came from soybean meal and 50% from menhaden fishmeal experienced increases in weight gain and FE upon prebiotic supplementation (Buentello *et al.* 2010). The effect vanished when all dietary protein originated from menhaden fishmeal. It would appear, therefore, as if beneficial effects of certain prebiotics on weight gain are most evident when diets have marginal nutrient bioavailability. In the present experiment, squid meal supplied a major portion of the dietary protein. This ingredient was proven to be highly digestible for shrimp (Buchanan *et al.* 1997). Therefore, it is possible that more dramatic results could be afforded by Previda® supplementation, if soybean meal or other plant proteins were to contribute in a larger measure to the overall dietary protein.

Data from the present study indicate that Previda® supplementation resulted in distinctly different microbial communities in the shrimp GIT compared with shrimp fed the basal diet. These results are consistent with those of Li *et al.* (2007), who observed that DGGE banding patterns of enteric bacteria found in the GIT of *L. vannamei* fed short-chain FOS also differed significantly from those of shrimp fed the control diets. Microbes present in the pond environment will invariably influence GIT communities in shrimp; therefore, more research is necessary to fully characterize the effects of Previda® supplementation over microbial communities in the guts of pond-raised shrimp.

According to Li *et al.* (2007) and Dimitroglou *et al.* (2011), although there are a limited number of publications on indigenous microbial communities inhabiting the GIT of shrimp, the interactive influences of microbial populations and shrimp health, as well as potential influences of diets supplemented with prebiotics, are generally not well understood. This poor understanding accounts in part for inconsistencies in results in fish and shrimp in regard to improving immune status of these animals following application of prebiotics. In general, prebiotic actions are believed to exert positive influences over the GIT milieu such that desirable microbial populations are favoured. Selected microbial populations foster disease prevention via niche competition, pH modifications and/or the release of natural antibiotics (Manning & Gibson 2004). In addition, these selected microbes may also enhance some components of the non-specific immune system (Smith *et al.* 1984; Sung *et al.* 1994; Qinghui *et al.* 2007). In the present study, levels of haemolymph protein and glucose were significantly reduced and levels of hemocyte phagocytic capacity and hyaline cell counts were significantly enhanced in shrimp fed 1.6 g kg^{-1} Previda® as compared with same indicators obtained for shrimp fed the basal diet. These measurements indicate a reduction in stress levels for shrimp and improvements in immune response (Mustafa *et al.* 2000; Hai & Fotedar 2009). Although under the present laboratory conditions, stress was adjudged minimal and no pathological organisms were evident in the culture media, it is known that the innate immune system comprises cells and mechanisms that defend the host from infection by other organisms in a non-specific manner. We argue that a Previda®-enhanced first line of defence would be available under commercial culture conditions where stressors abound and pathogenic organisms are not uncommon. These observations would need to be independently confirmed in challenge trials under practical conditions.

Another way in which Previda® supplementation could benefit commercial shrimp culture is by preventing attachment of pathogenic bacteria to the intestinal cell wall in cultured organisms. According to the manufacturer, mannan is the most prevalent oligosaccharide in Previda®. It is well documented that mannans, and possibly other oligosaccharides, serve as alternate attachment sites for Gram-negative pathogens, thereby preventing attachment onto enterocytes and subsequent enteric infection. Adherence of the pathogenic microbe to the enterocyte cell wall is thought to be a prerequisite for the onset of infection (Gibbons & Van Houte 1975). In the present experiment, shrimp fed diets supplemented with Previda® at 0.5 g kg^{-1} and 1.6 g kg^{-1}, respectively, had improved phagocytic capacity of hemocytes relative to those fed the basal diet. In the shrimp innate immunity system, phagocytosis is the essential mechanism to eliminate the invading microorganisms and other kinds of particles by degradation of internalized pathogens via the participation of lytic enzymes and the oxidase complex (Liu *et al.* 2009). That phagocytosis was enhanced upon Previda® supplementation in the present study is further corroborated by a significant increase ($P = 0.03$) in hyaline cells – which are chiefly involved in phagocytosis (Li *et al.* 2010). Further studies are suggested, especially those focused on characterizing and identifying the diverse microbial populations which contributed to the banding patterns revealed by DGGE. Such studies which should be conducted in facilities where shrimp are challenged with known pathogens will help to identify the beneficial or good microbiota which may have contributed to enhanced stress capabilities revealed in the present study.

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References

Anuta, J.D., Buentello, A., Patnaik, S., Lawrence, A.L., Mustafa, A., Hume, M.E., Gatlin, D.M. III & Kemp, M.C. (2011) Effect of dietary supplementation of acidic calcium sulfate (Vitoxal) on growth, survival, immune response and gut microbiota of the Pacific white shrimp, *Litopenaeus vannamei*. *J. World Aquac. Soc.*, **42**, 834–844.

Bailey, J., Blakenship, L. & Cox, N. (1991) Effect of fructooligosaccharide on Salmonella colonization of the chicken intestine. *Poult. Sci.*, **70**, 2433–2438.

Braak, K. (2002) Haemocytic defence in black tiger shrimp. Ph.D. thesis, Wageningen University, The Netherlands.

Brown, L.L., Iwama, G.K. & Evelyn, T.P.T. (1996) The effects of early exposure of Coho salmon (*Oncorhynchus kisutch)* eggs to the p57 protein of *Renibacterium salmoninarum* on the development of immunity to the pathogen. *Fish Shellfish Immunol.*, **6**, 149–165.

Buchanan, J., Sarac, H.Z., Poppa, D. & Cowan, R.T. (1997) Effects of enzyme addition to canola meal in prawn diets. *Aquaculture*, **151**, 29–35.

Buentello, J.A., Neill, W.H. & Gatlin, D.M. III (2010) Effects of dietary prebiotics on growth, feed efficiency and non-specific immunity of juvenile red drum *Sciaenops ocellatus* fed soybean-based diets. *Aquacult. Res.*, **41**, 411–418.

D'Abramo, L.R.D., Conklin, D.E. & Akiyama, D.M. (1997) Crustacean Nutrition. World Aquaculture Society, Baton Rouge, LA.

Dimitroglou, A., Merrifield, D.L., Carnevali, O., Picchieti, S., Avella, M., Daniels, C., Guroy, D. & Davies, S.J. (2011) Microbial manipulations to improve fish health and production – a Mediterranean perspective. *Fish Shellfish Immunol.*, **30**, 1–16.

Enane, N.A., Frenkel, K., O'Connor, J.M., Squibb, K.S. & Zelikoff, J.T. (1993) Biological markers of MØ activation: application for fish phagocytes. *Immunology*, **80**, 68–72.

Genc, M.A., Aktas, M., Genc, E. & Yilmaz, E. (2007) Effects of dietary mannan oligosaccharide on growth, body composition and hepatopancreas histology of *Penaeus semisulcatus* (de Haan 1844). *Aquacult. Nutr.*, **13**, 156–161.

Gensic, M., Wissing, P., Keefe, R. & Mustafa, A. (2004) Effects of iodized feed on stress modulation in steelhead trout, *Oncorhynchus mykiss* (Walbaum). *Aquacult. Res.*, **35**, 1117–1121.

Gibbons, R.J. & Van Houte, J. (1975) Bacterial adherence in oral microbial ecology. *Ann. Rev. Microbiol.*, **29**, 19–44.

Gong, H., Lawrence, A.L., Jiang, D., Castille, F.L. & Gatlin, D.M. III (2000) Lipid nutrition of juvenile *Litopenaeus vannamei*: I. Dietary cholesterol and de-oiled soy lecithin requirements and their interaction. *Aquaculture*, **190**, 305–324.

Grisdale-Helland, B., Helland, S.J. & Gatlin, D.M. III (2008) The effects of dietary supplementation with mannanoligosaccharide, fructooligosaccharide or galactooligosaccharide on the growth and feed utilization of Atlantic salmon (*Salmon salar*) fed diets with soybean meal or inulin with or without addition of antibiotics. *Aquaculture*, **261**, 392–406.

Gultepe, N., Hisar, O., Salnur, S., Hossu, B., Tanrikul, T.T. & Aydin, S. (2012) Preliminary assessment of dietary mannanoligosaccharides on growth performance and health status of Gilthead seabream (*Sparus auratus*). *J. Aquat. Anim. Health*, **24**, 37–42.

Hai, N. & Fotedar, R. (2009) Comparison of the effects of the prebiotics (Bio-Moss and B-1,3-D-glucan) and the customized probiotics (*Pseudomonas synxantha and P. aeruginosa*) on the culture of juvenile western king prawns (*Penaeus latisulcatus* Kishinouye, 1896). *Aquaculture*, **289**, 310–316.

Hume, M.E., Kubena, L.F., Edrington, T.S., Donskey, C.J., Moore, R.W., Ricke, S.C. & Nisbet, D.J. (2003) Poultry digestive microflora biodiversity as indicated by denaturing gradient gel electrophoresis. *Poult. Sci.*, **82**, 1100–1107.

Kihara, M., Ohba, K. & Sakata, T. (1995) Trophic effect of dietary lactosucrose on intestinal tunica muscularis and utilization of this sugar by gut microbes in red sea bream, *Pagrus major*, a marine carnivorous teleost, under artificial rearing. *Comp. Biochem. Physiol.*, **112**, 629–634.

Lara-Flores, M., Olvera- Novoa, M.A., Guzman- Mendez, B.E. & Lopez-Madrid, W. (2003) Use of the bacteria *Streptococcus faecium* and *Lactobacillus acidophilus* and the yeast *Saccharomyces cerevisiae* as growth promoters in Nile tilapia (*Oreochromis niloticus*). *Aquaculture*, **216**, 193–201.

Li, P. & Gatlin, D.M. III (2004) Dietary brewer's yeast and the prebiotic Grobiotic-A influence growth performance, immune responses and resistance of hybrid striped bass (*Morone chrysops x M. saxatilis)* to *Streptococcus iniae* infection. *Aquaculture*, **231**, 445–456.

Li, P., Lewis, D.H. & Gatlin, D.M. III (2004a) Dietary oligonucleotide from yeast RNA influences immune responses and resistance of hybrid striped bass (*Morone chrysops* x *M. saxatilis*) to *Streptococcus iniae* infection. *Fish Shellfish Immunol.*, **16**, 561–569.

Li, P., Wang, X. & Gatlin, D.M. III (2004b) Excessive dietary levamisole suppresses growth performance of hybrid striped bass (*Morone chrysops* x *M. saxatilis*), and elevated levamisole in vitro impairs macrophage function. *Aquacult. Res.*, **35**, 1380–1383.

Li, P., Burr, G.S., Gatlin, D.M. III, Hume, M.E., Patnaik, S., Castille, F.L. & Lawrence, A.L. (2007) Dietary supplementation of short- chain fructooligosaccharides influences gastrointestinal microbiota composition and immunity characteristics of Pacific White Shrimp *Litopenaeus vannamei* cultured in a recirculating system. *J. Nutr.*, **137**, 2763–2768.

Li, J., Tan, B. & Mai, K. (2009) Dietary probiotic *Bacillus* OJ and isomaltooligosaccharides influence the intestinal microbial population, immune responses and resistance to white spot syndrome virus in shrimp (*Litopenaeus vannamei*). *Aquaculture*, **291**, 35–40.

Li, C.-C., Yeh, S.-T. & Chen, J.-C. (2010) Innate immunity of the white shrimp *Litopenaeus vannamei* weakened by the combination of a *Vibrio alginolyticus* injection and low-salinity stress. *Fish Shellfish Immunol.*, **28**, 121–127.

Liu, W., Han, F. & Zhang, X. (2009) Ran GTPase regulates hemocytic phagocytosis of shrimp by interaction with myosin. *J. Prot. Res.*, **8**, 1198–1206.

Lückstädt, C. (2008) The use of acidifiers in fish nutrition. *Persp. Agri. Vet. Sci. Nutr. Nat. Res*, **3**, 1–8.

Mahious, A.S., Gatesoupe, F.J., Herri, M., Metailler, R. & Ollevier, F. (2006) Effects of dietary inulin and oligosaccharides as prebiotics for weaning turbot *Psetta maxima* (Linnaeus, C. 1758). *Aquacult. Int.*, **14**, 219–229.

Manning, T.S. & Gibson, G.R. (2004) Prebiotics. *Best Pract. Res. Clini. Gastr.*, **18**, 287–298.

Mustafa, A., MacWilliams, C., Fernandez, N., Matchett, K., Conboy, G. & Burka, J. (2000) Effects of sea lice (*Lepeophtheirus salmonis* Kroyer, 1837) infestation on macrophage functions in Atlantic salmon (*Salmo salar* L.). *Fish Shellfish Immunol.*, **10**, 47–59.

Ng, S.C., Hart, A.L., Kamm, M.A., Stagg, A.J. & Knight, S.C. (2009) Mechanisms of action of probiotics: recent advances. *Inflamm. Bow. Dis.*, **15**, 300–310.

Probert, H.M., Apajalahti, J.H.A., Rautonen, N., Stowell, J. & Gibson, G.R. (2004) Polydextrose lactitol, and fructooligosaccharides fermentation by colonic bacteria in a three-stage continuous culture system. *Appl. Environ. Microbiol.*, **70**, 4505–4511.

Qinghui, A., Mai, K., Zhang, L., Tan, B., Zhang, W., Xu, W. & Li, H. (2007) Effects of dietary β-1, 3 glucan on innate immune response of large yellow croaker, *Pseudosciaena crocea*. *Fish Shellfish Immunol.*, **22**, 394–402.

Refstie, S., Bakke- McKellep, A.M., Penn, M.H., Sundby, A., Shearer, K.D., Krogdahl, A. & A., (2006) Capacity for digestive hydrolysis and amino acid absorption in Atlantic salmon (*Salmo salar*) fed diets with soybean meal or inulin with or without addition of antibiotics. *Aquaculture*, **261**, 392–406.

Ringø, E., Sperstad, S., Myklebust, R., Mayhew, T.M. & Olsen, R.E. (2006) The effect of dietary inulin on bacteria associated with hindgut of Arctic charr (*Salvelinus alpinus* L.). *Aquacult. Res.*, **37**, 891–897.

Ringø, E., Løvmo, L., Kristiansen, M., Bakken, Y., Salinas, I., Myklebust, R., Olsen, R.E. & Mayhew, T.M. (2010) Lactic acid bacteria vs. pathogens in the gastrointestinal tract of fish: a review. *Aquacult. Res.*, **41**, 451–467.

Rodrigues-Estrada, U., Satoh, S., Haga, Y., Fushimi, H. & Sweetman, J. (2008) Studies of the effects of mannanoligosaccharides, *Enterococcus faecalis*, and polyhydrobutyric acid as immune stimulant and growth promoting ingredients in rainbow trout diets. 5th World Fisheries Congress, Yokohama, Japan, October 20–25. Abstract 2d-1-5.

Sajeevan, T.P., Philip, R. & Bright Singh, I.S. (2006) Immunostimulating effect of a marine yeast *Candida sake* S165 in *Fenneropenaeus indicus*. *Aquaculture*, **257**, 150–155.

Schreck, C.B. & Moyle, P.B. eds (1990) Methods for Fish Biology. American Fisheries Society, Bethesda, MD.

Smith, V.J., Soderhall, K. & Hamilton, M. (1984) β-1, 3-glucan induced cellular defense reactions in the shore crab, *Carcinus maenas*. *Comp. Biochem. Physiol.*, **77A**, 635–639.

Smith, V.J., Brown, J.H. & Hauton, C. (2003) Immunostimulation in crustaceans: does it really protect against infection? *Fish Shellfish Immunol.*, **15**, 71–90.

Spotte, S. (1979) Fish and Invertebrate Culture: Water Management in Closed Systems, 2nd edn. Wiley, New York, NY, USA.

Staykov, Y., Spring, P., Denev, S. & Sweetman, J. (2007) Effect of a manna oligosaccharide on the growth performance and immune status of rainbow trout (*Oncorhynchus mykiss*). *Aquacul. Int.*, **15**, 153–161.

Sung, H.H., Kou, G.H. & Song, Y.L. (1994) Vibriosis resistance induced by glucan treatment in tiger shrimp (*P. monodon*). *Fish Pathol.*, **29**, 11–17.

Torrecillas, S., Makol, A., Caballero, M.J., Montero, D., Robaina, L., Real, F., Sweetman, J., Tort, L. & Izquierdo, M.S. (2007) Immune stimulation and improved infection resistance in European sea bass (*Dicentrarchus labrax*) fed mannan-oligosaccharides. *Fish Shellfish Immunol.*, **23**, 969–981.

Wang, Y., Jian- Rong, L. & Junda, L. (2008) Probiotics in aquaculture. *Aquaculture*, **281**, 1–4.

Wedemeyer, G.A., Barton, B. & McLeay, D. (1990) Stress and acclimation. In: Methods for Fish Biology (Schreck, C. & Moyle, P.B. ed.), pp. 451–489. American Fisheries Society, Bethesda, MD, USA.

Zhou, Z., Ding, Z. & Huiyuan, L.U. (2007) Effects of dietary short-chain fructooligosaccharides on intestinal microflora, survival and growth performance of juvenile white shrimp, *Litopenaeus vannamei*. *J. World Aquac. Soc.*, **38**, 296–306.